Identification of HSP16.9 gene in local wheat genotypes using an allele-specific primer

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Plants have developed various mechanisms in heat-stress adaptation,including changes in protein metabolism such as the induction of heat shock proteins (HSPs). Using the SNP based allele specific primer, Azerbaijani wheat germplasm was analyzed for HSP 16.9 gene belonging to low molecular weight heat shock proteins.The analysis of the electrophoretic profiles of PCR results showed that 197 bp fragment, which is considered to be diagnostic for HSP 16.9 gene, was synthesized in 73% of the studied genotypes (36 genotypes of bread (*Triticum aestivum* L.) and 15 genotypes of durum (*Triticum durum* Desf.) wheat. The expected fragment was not amplified in 6 samples of durum and 9 of bread wheat genotypes.

Keywords: *Wheat genotypes, heat stress, heat shock proteins, HSP16.9, PCR, allele-specific primer*

INTRODUCTION

Due to predicted global warming improving tolerance of food plants to high temperature is considered to be a serious issue for sustainable agriculture worldwide (Xu et al., 2011). High extreme temperatures during climate change directly impact on plant productivity, respiration, reproduction, growth and other biochemical processes (Hatfield & Prueger,2015). Extreme heat during stress can damage intermolecular interactions that needed for growth, thus impairing plant development (Bita & Gerats, 2013). In order to tolerate such stressful conditions plants worked out response mechanisms such as: root growth to more deeply wet parts of soil, closed stomata in order to prevent water loss during transpiration, stabilization of cell membrane, reorganization of the genetic apparatus to regulate the synthesis of stress proteins (Nandha et al., 2018). In other words, various mechanisms, including changes at the molecular, cellular, biochemical, physiological, and whole-plant levels exist for achieving plant tolerance to high temperature (Hasanuzzaman et al., 2013; Ahenger et al., 2017). It was identified, that at biochemical level, heat-labile proteins exposed to denaturation under high temperature conditions and significant increase in level of harmful reactive oxygen species (ROS) in plant cells (Mittler et al., 2012; Grover et al., 2013). Heat stress significantly affects protein metabolism, including degradation of proteins, inhibition of protein accumulation, and induction of certain protein synthesis, depending on the level and duration of heat stress (Monjardino et al., 2005, He and Huang, 2007). Downregulation of proteins functioning in lipid biogenesis, cytoskeleton structure, sulfate assimilation, amino acid biosynthesis, nuclear transport and antioxidant response are involved in the moderate heat response (Ferreira et al., 2006). The transcription and translation of a small set of proteins, called heat shock proteins (HSPs) may be induced or enhanced when plants are exposed to elevated temperatures because the synthesis of most normal proteins and mRNAs are inhibited under heat stress (Al-Whaibi et al., 2011; Wang et al., 2017).

HSPs are known as a stress proteins and synthesized in response to all kind of stress such as: high temperature, UV radiation, heavy metal ions, and exposure to toxic or infectious agents (Urban-Chmielet al., 2013). Stressing agents lead to an immediate block of every important metabolic process, including DNA replication, transcription, mRNA export, and translation, until

the cells recover (Biamonti and Caceres, 2009). HSPs function as a molecular chaperones which are responsible for protein folding, assembly, translocation and degradation in many cellular processes (Wang et al., 2004). Expression of HSPs is regulated by heat shock transcription factors (HSF).

Wheat is the most important cereal crop and always exposed to various abiotic stress. Currently, in many regions of the world wheat crop is exposed to heat stress during grain filling period and thus adversely affecting the plant growth, yield and grain quality (Garg et al., 2012; Xue et al., 2013). Extreme heat conditions during late growth stage is a problem in 40% of the wheat growing areas. Studies showed that heat tolerant wheat cultivars demonstrated strong and various response to heat stress in the form of HSPs compared to susceptible cultivars (Skylas et al., 2002). In wheat genotype, exposed to heat stress were detected mRNA encoding major class of low molecular weight HSPs (HSP 16.9) (Nguyen et al., 1994; Garg et al., 2012). According to some reports different types of HSP are synthesized in different tissues of wheat in response to duration and kind of heat stress (Zivy, 1987; Weng and Nguyen, 1992; Treglia et al., 1999; Rampino et al., 2009; Sharma-Natu et al., 2010; Xu et al., 2011).

The study aimed at analyzinggermplasm of Azerbaijani wheat genotypes, for HSP 16.9 gene belonging to low molecular weight heat shock proteins, using the SNP based allele specific primer.

MATERIALS AND METHODS

Plant Materials

Wheat genotypes (36 genotypes of bread (*Triticum aestivum* L.) and 15 genotypes of durum (*Triticum durum* Desf.) wheat) genotypes collected in the Gene Pool of the Research Institute of Agriculture (Baku) acted as a research object. Plants were cultivated in field conditions.

Extraction of Plant DNA

DNA extraction was carried out using the CTAB method with some modifications (Murray and Thompson, 1980). Fresh plant tissue as a fragment of leaf was minced in liquid nitrogen, suspended in 1000 µl of CTAB extraction buffer (100 mM Tris-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 mM NaCl; 40 mMβ-mercaptoethanol), and pre-warmed in a water bath at 60ºC. Homogenization was completed by intense Vortex shaking. Then 400 ml of chloroform (99.8%) was added into each tube and the tubes were gently mixed. Next the tubes were placed in a water bath and incubated for 10 min at 60ºC. After incubation, the tubes were centrifuged in an Eppendorf type benchtop centrifuge (15,000 g) for 10 min at room temperature. After centrifugation the supernatant was carefully selected (taking care not to capture sediment particles) and transferred to clean 1.5 ml Eppendorf type tubes and 600 ml of cold isopropanol was added, mixed well and left at room temperature for 3-5 minutes. At this stage we can observe the dispersed DNA precipitate. The tube contents were centrifuged at room temperature in the Eppendorf type benchtop centrifuge (15,000 g) for 10 min.

The precipitate was washed several times with 70% ethanol, dried in a thermostat at 56ºC for 5 minutes and dissolved in TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA). Samples were left in a refrigerator at 4ºC for the complete dissolution of the DNA in a buffer.

DNA Quantification

After dissolution of the DNA the quantity was determined by optical density (OD) at λ=260 using the ULTROSPEC 3300 PRO spectrophotometer ("AMERSHAM", USA).

Purity of the genomic DNA was determined by the ratio of absorptions at A260/A280. Quality of the DNA was checked on the basis of performance of the extracted DNA samples in 0.8% agarose gel stained with 10 mg / ml of ethidium bromide in 1 × TBE (Tris base, Boric acid, EDTA) buffer. The gel was developed and photographed under ultraviolet light using "Gel Documentation System UVITEK" (UK).

DNA Amplification

Polymerase chain reaction was performed by Williams (1990). DNA amplification was performed in a 25 µl reaction mixture volume, containing 10 × buffer, 20 ng of the genomic DNA, 0.2 µM primer, 200 µM of each of the following: dATP, dCTP, dGTP and dTTP, 2,5 mM $MgCl_2$, and 0.2 units of Taq-polymerase in the incubation buffer. An allele-specific primer pair indicated in the Table 1. were used for HSP16.9 gene screening.

Table 1. Primer sequences for the markers linked to the HSP16.9 gene

DNA marker	Nucleotide sequences (5′→3′)	Expected fragment, bp
HSP16.9F	CAGCAATCAACACCACGATG	197
HSP16.9R	TGCCACTTGTCGTTCTTGTC	

PCR was performed in the "Applied Biosystems 2720 Thermal Cycler" (Singapore) thermocycler under the following conditions: 1 cycle - 3 minutes at 94 ºC; 38 cycles - 1 min at 94 ºC, an annealing step at variable annealing temperatures depending on the primer pairs for 1 min, 2 minutes at 72 ºC; the final elongation cycle was performed at 72 °C for 10 min, then kept at 4°C.

The reaction products were separated by electrophoresis in a 3% agarose gel in the HR-2025-High Resolution («IBI SCIENTIFIC» U.S.) horizontal electrophoresis machine with addition of ethidium bromide and documented using «Gel Documentation System UVITEK». Dimensions of amplified fragments were determined with respect to 1kb DNA marker. Statistical analysis included binary matrix compilation for each of the primers, in which "presence" (1) or "absence" (0) of fragments with equal molecular weight on the electrophoregram were noted.

RESULTS AND DISCUSSION

Tolerance to heat stress is a complex phenomenon controlled by multiple genes responsible for a number of physiological and biochemical changes and the mechanism of heat tolerance cannot be explained by a single trait. Germoplasm of 36 bread (*Triticum aestivum* L.) and 15 durum (*Triticum durum* Desf.) wheat genotypes from the wheat genefund of the Research Institute of Crop Husbandry was evaluated for HSP 16.9 gene belonging to low molecular weight heat shock proteins. To this end, allele-specific primers based on SNP were designed to screen the other heat tolerant and susceptible genotypes. The analysis of the electrophoretic profiles of PCR results showed that 197 bp fragment, which is considered to be diagnostic for HSP 16.9 gene, was successfully synthesized in 73% of the studied genotypes (Figure).

Amplification of HSP gene-specific fragments was found in approximately 60% of durum and 77% of bread wheat genotypes. In 27% of all genotypes (6 samples of durum and 9 of bread wheat genotypes) the expected fragment was not amplified.

In physiological conditions unrevealed HSPs were detected in stress period produced upon to plant tolerance to stress, involving drought, salinity, high temperature and etc. (Low et al., 2000; Hamilton and Heckathorn, 2001; Scharf et al., 2001; Zhang et al., 2008). The main role of HSPs is to function as molecular chaperones. Chaperones are the proteins which regulate protein folding, assist to newly synthesized proteins achieve their native state also they prevent proteins non-specific aggregation and take part in protein refolding under thermal stress conditions (Feder & Hofmann, 1999; Schulze-Lefert, 2004; Panaretou & Zhai, 2008; Gupta et al., 2010). Nevertheless, the precise role of HSPs in regulating the plant molecular mechanisms responsible for normal growth and development, and stress response has to be clarified (Xu et al., 2011).

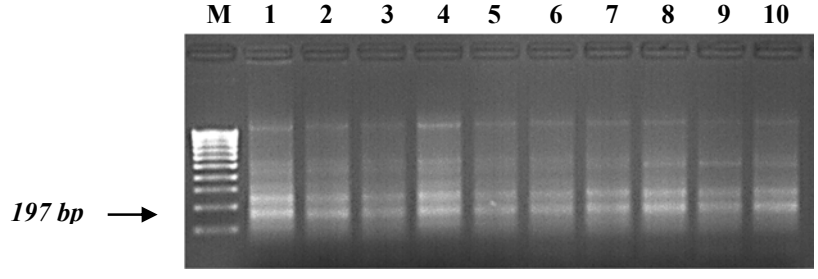

Fig. PCR-profiles of wheat plants for HSP16.9. M - 100 bp DNA ladder, 1. Dagdash, 2. Farandole, 3. Azeri, 4. Tale-38, 5. Nurlu-99, 6. Garabag, 7. Ag bugda, 8. Tigre, 9. Agali, 10. Renan, 11. Zirva-85, 12. Layagatli-80, 13. Murov; Arrow shows ~ 197 bp band.

Table 2. Results of the PCR analysis for HSP16.9 gene. [+] – presence of the expected locus, [-] – absence of this locus.

Triticum aestivum L.			
Genotype	HSP16.9 (197 bp)	Genotype	HSP16.9 (197 bp)
Dagdash	+	Pirshahin-1	+
Gyrmyzy gul	-	Parzivan-2	+
Sevinj	-	Gyzyl bugda	-
Fatima	+	1st WWEERYT4	+
11th Fawwon N22	+	12th Fawwon	+
Farandole	+	Azamatli-95	+
Azeri	+	4th FEFWSN	+
Parzivan-1	-	Murov-2	+
Tale-38	+	Qualite	+
Nurlu-99	+	Gunashli	+
Taraggi	+	Ruzi-84	+
Shaki-1	+	Ugur	+
Agali	+	Mirbashir-128	+
Renan	+	Aran	-
Saratovskaya-29	+	Farahim-2012	-
Zirva-85	+	Shafag-2	-
Layagatli-80	+	Fransa	-
Murov	+	A2	+
Triticum durum Desf.			
Sharg	-	Shiraslan-23	+
Mirbashir-50	+	Vugar	-
Barakatli-95	-	Ag-bugda	+
Gyrmyzy bugda	-	Tigre	+
Shirvan -3	+	Garagylchyg-2	-
Alinja-84	+	Turan	+
Kakhraba	+	Mugan	-
Garabag	+		

In plants, HSPs are classified into five principal classes, namely, HSP100, HSP90, HSP70/DnaK, HSP60/GroE and small heat shock proteins (sHSP) based on their molecular weight (Wang et al., 2004). In order to delineate the molecular roles of these HSPs, several studies on identification and characterization of HSPs and their corresponding genes were performed in plant species such as Arabidopsis, tomato and rice (Hu et al., 2009; Sung et al., 2001; Siddique et al., 2008; Scharf et al., 2001). In rice, 10, 9, 26 and 29 HSPs were identified belonging to HSP100, HSP90, HSP70, and sHSPs, respectively. Expression profiling of these HSP encoding genes in response to heat, cold, drought and salt stresses showed their differential expression with significant upregulation of sHSP genes during heat stress6. Identification and expression profiling of sHSP genes in barley during drought stress was reported by Reddy et. al (2014). The study identified 20 sHSPs, which are shown to be differentially regulated in response to drought stress (Singh et al., 2016).

In particular, sHSP has a much larger binding stoichiometry than other molecular chaperones, indicating that sHSP functions as a reservoir to stabilize the flood of denatured proteins in response to stress (Xu et al., 2011). Heat-induced oligomer dissociation is suggested to be a major mechanism by which plant sHSPs can expose normally inaccessible, hydrophobic client-binding surfaces. However, there is not sufficient information on the interactions between sHSP and nonnative proteins and how these nonnative proteins are subsequently refolded. This may be explained by limited knowledge of the molecular structure of sHSPs (Haslbeck et al., 2005). Wheat TaHsp16.9-CI (wHSP16.9, PDB Id: 1GME) is one of the few solved crystallographic structures of sHSPs.The basic building block of wHSP16.9 is found to be a dimer, which further assembles as a 12-mer consisting of two trimers of dimers. In solution, wHSP16.9 can dissociate into smaller oligomeric states in a temperature dependent manner. The hydrophobic patches buried in the oligomeric interface are likely to be exposed to heat-induced dissociation of sHSP oligomers, resulting in binding and stabilization of denatured proteins (Van Montfort et al., 2011). Presence of a carboxyl terminal called heat-shock domain is a characteristic pattern of these proteins (Helm et al., 1993). There are six classes of genes that encode HSPs. Classification was according to sequence similarity and the location of these proteins in cell. In cytoplasm were identified 2 classes of proteins which encoded by two group of genes (Vierling, 1991; Waters et al., 1996). In non-stress conditions expression of these genes is limited and appears at embryogenesis, germination stage of development also during development of pollen grains, and fruit ripening (Sun et al., 2002). Expression of these genes is regulated by proteins called heat shock factors (Hsfs) which are present in cytoplasm in an inactive form. These factors are the transcriptional activators for HSPs (Baniwal et al., 2004; Hu et al., 2009).

Thus, a significant role in conferring abiotic stress tolerance to crop plants belongs to heat shock

proteins (HSPs). Therefore, HSPs and their encoding genes have been extensively characterized in several plant species. However, for understanding their precise role in the molecular mechanisms of stress response, it is necessary to investigate structure, organization, evolution and expression profiling of naturally stress tolerant crops.

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Yerli buğda genotıplərındə allel-spesifik praymerlə HSP16.9 geninin identifikasiyası

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Bitkilərdə istilik stresinə qarşı müxtəlif, o cümlədən istilik şoku zülallarının (HSPs) induksiyası kimi zülal metabolizmində baş verən dəyişiklikləri əhatə edən mudafiə mexanizmləri formalaşmışdır. SNP əsaslı allel-spesifik praymerdən istifadə etməklə Azərbaycanın buğda genotiplərində aşağı molekul ktləli istilik şoku zülallarına aid HSP 16.9 geninin analizi aparılmışdır. PZR nəticələrinin elektroforetik profillərinə əsasən HSP 16.9 üçün diaqnostik hesab olunan 197 bp fraqmenti tədqiq edilmiş genotiplərin 73%-də (36 yumşaq buğda (*Triticum aestivum* L.) və 15 bərk buğda (*Triticum durum* Desf.) genotipləri) sintez edilmişdir. 6 bərk və 9 yumşaq buğda genotipində gözlənilən fraqment amplifikasiya olunmamışdır.

Açar sözlər: *Buğda genotipləri, istilik stresi, istilik şoku zülalları, HSP16,9, PZR, allel-spesifik praymer*